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January 24, 2005

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APPLICATION NUMBER: *60/610,474*
FILING DATE: *September 16, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US04/41282*

Certified by

Jon W. Dudas

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PTO/SB/16 (08-03)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 509623664 US

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Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Live Avirulent Microbes As Vaccines For Anthrax and Plague

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 10 ☐ CD(s), Number

☑ Drawing(s) *Number of Sheets* 15 ☐ Other (specify)

☐ Application Date Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees. (check no. 5399)

☐ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-0268

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): 160.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☑ Yes, the name of the U.S. Government agency and the Government contract number are: Department of Defense (JVAP), subcontract under DynPort Vaccine Co., LLC, no. DPSC-02-02257

Respectfully submitted,

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Date September 16, 2004

REGISTRATION NO. 30,237 *(if appropriate)*

Docket Number: AVA-440.2 PRV

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/16 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Docket Number AVA-440.2 PRV

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Number 1 of 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Sizemore et al.	
Serial No.:	(not yet assigned)	Examiner:
Filed:	(concurrently herewith)	Art Unit:
Entitled:	Live Avirulent Microbes As Vaccines for Anthrax and Plague	

Atty. Docket No.: AVA-440.2 PRV

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF EXPRESS MAIL

The undersigned hereby certifies that this certificate and the papers and fees identified below as being transmitted herewith are being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated below and are addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

The following items are transmitted herewith:

1. Provisional Application For Patent Coversheet (2 pages) (in duplicate)
2. Specification (10 pages)
3. Drawings (15 sheets)
4. Check No. 5399 in the amount of $ 160.00 to cover provisional patent application filing fee.
5. Return Postal Card

Express Mail # EV 509623664 US

date of deposit: September 16, 2004

Nasim G. Memon

LIVE AVIRULENT MICROBES AS VACCINES FOR ANTHRAX AND PLAGUE

FIELD OF THE INVENTION

[0001] The present invention relates to live attenuated bacterial vectors to deliver protective antigens for eliciting an immune response in a mammal against *B. anthracis* and/or *Y. pestis*.

BACKGROUND OF THE INVENTION

[0002] Anthrax is an infectious bacterial disease caused by *Bacillis anthracis*. It occurs most commonly in wild and domestic herbivores (sheep, goats, camels, antelope, cattle, etc.) but may also occur in humans. Infection can occur by cutaneous exposure, by ingestion (gastrointestinal anthrax), or by inhalation (pulmonary anthrax). 95% of anthrax infections in humans occur by cutaneous infection, either from contact with unvaccinated, infected animals in an agricultural setting, or by handling contaminated animal products (meat, leather, hides, hair, wool, etc.) in an industrial setting.

[0003] Cutaneous anthrax is fatal in about 20% of cases if untreated, but it can usually be overcome with appropriate antimicrobial therapy. Inhalation or gastrointestinal anthrax infection is much more serious and much more difficult to treat. Inhalation anthrax results in repiratory shock and is fatal in 90%-100% of cases; gastrointestinal anthrax results in severe fever, nausea and vomiting, resulting in death in 25%-75% of cases.

[0004] An effective vaccine against anthrax was developed in the United States in the 1950s and 1960s, and a vaccine was approved by the FDA in 1970.

[0005] In recent years the threat of airborn transmission of anthrax has been thought to increase as *B. anthracis* was identified as a possible agent for biological warfare. (See, e.g., U.S. Congress, Office of Technology Assessment, *Proliferation of Weapons of Mass Destruction: Assessing the Risks*, OTA-ISC-559 (Washington, D.C.; U.S. Government Printing Office, August 1993); www.anthrax.osd.mil.) This

threat has now been realized in the past few years in the form of mailed anthrax spores, resulting in several deaths. Whereas historically only individuals at high risk, such as veterinarians, livestock handlers, wool shearers, abbatoire workers, etc., needed to consider being vaccinated, the threat to military personnel of the possibility of biological weapons deployment caused the United States military to adopt a sweeping anthrax vaccination program in 1997, under which it was intended to administer the anthrax vaccine to 2.4 million military personnel in all branches of service. (See, e.g., Secretary of Defense, Memorandum for Secretaries of the Military Departments et al., May 18, 1998, *Implementation of the Anthrax Vaccination Program for the Total Force.*)

[0006] The only mass produced anthrax vaccine, Anthrax Vaccine Adsorbed (or AVA, commercial name BioThrax™), is a noninfectious sterile filtrate of an attenuated strain of *B. anthracis*, adsorbed to aluminum hydroxide (alum) adjuvant, with ≤ 0.02% formaldehyde and 0.0025% benzethonium chloride added. (Friedlander et al., *JAMA*, 282(22):2104-2106 (1999).) The course of vaccination consists of six subcutaneous injections of 0.5 mL doses of vaccine over eighteen months, with annual boosters to maintain immunity. This vaccination is believed to provide immunity that is 90%-100% effective against aerosol anthrax challenge, based on animal studies and incidental human data. (Friedlander et al., *id.*)

[0007] While the AVA is effective, the vaccine strain employed (i.e., a non-proteolytic, non-capsulated mutant strain of *B. anthracis*, V770-NP1-R) has some disadvantageous characteristics: Despite its mutations, the strain retains a sporogenic and fully toxogenic phenotype, and use of the whole strain in vaccine production results in lot-to-lot variability in levels of Protective Antigen, as well as inclusion of PA degradation products and other bacterial products, which may include EF and LF. (Farchaus, J., et al., *Applied & Environmental Microbiol.*, 64(3):982-991 (1998).)

[0008] Plague is caused by the Gram-negative bacterium, *Yersinia pestis,* and is one of the oldest documented infectious diseases. Plague manifests in humans in bubonic or pneumonic form, depending on the route of transmission (flea bite or airborne, respectively) and the systems affected (cutaneous or pulmonary, respectively). Inhalation anthrax and pneumonic plague are the most serious forms of anthrax and plague disease and thus aerosolization would likely be the manner employed to administer the weaponized form of these agents. The lethal route of each pathogen initiates infection at the mucosal surface of the respiratory tract.

[0009] Traditional vaccine approaches have focused on parenteral vaccination, which principally elicits the production of systemic antibody (immunoglobulin G, IgG) and not mucosal antibody (sIgA). Pre-clinical immunogenicity and efficacy studies evaluating the current anthrax vaccine (Anthrax Vaccine Adsorbed, or AVA), suggest that the presence of protective antigen (PA)-specific IgG correlates with

protection. Additional studies have demonstrated that functional antibody, capable of neutralizing PA activity *in vitro* is also a reliable surrogate marker for protection. Pre-clinical immunogenicity and efficacy studies evaluating candidate plague vaccines (based on *Yersinia pestis* F1 capsule or V antigen) have demonstrated that serum IgG is a reliable correlate of protection against experimental plague challenge, although T-lymphocyte responses may also contribute to protective immunity. The FDA-approved anthrax vaccine requires a dosing regimen that requires six injections over eighteen months. The vaccine takes several months to induce protective immunity and is reported to elicit undesirable reactions in a large number of vaccinees. For their part, candidate plague vaccines share the requirement of a multi-dose injection regimen and do not provide reliable protection against the pneumonic form of the disease.

[0010] During the Cold War, the United State and Soviet biological weapons programs developed techniques to aerosolize *Y. pestis* with the intent of facilitating the dissemination of the Plague bacterium. There is currently no commercially available vaccine against either cutaneous (bubonic) or respiratory (pneumonic) forms of Plague. An injectable, formaldehyde-killed *Y. pestis* whole cell vaccine was available, until recently, to vaccinate military and laboratory personnel at risk, and provided partial protection against bubonic plague. The vaccine's capacity to protect against pneumonic Plague was questionable however, an important consideration for a vaccine intended to protect against an airborne threat. The ability of this vaccine to protect against Pneumonic plague was not surprising, given that most parenterally administered vaccines elicit the production of serum (immunoglobulin G) and not mucosal (secretory immunoglobulin A) antibodies. This directly and adversely affects the readiness of our armed forces and homeland defenders.

[0011] In view of this background, there is a need for improved methods and vaccine compositions for immunization against anthrax and/or plague that are effective to raise an immune response against *B. anthracis* and/or *Y. pestis* but without generating unwanted side effects. These needs are addressed by the present invention, disclosed herein.

SUMMARY OF THE INVENTION

[0012] Efficacy studies evaluating anthrax and plague vaccines in animals have shown that antibodies specific for "Protective Antigen" or PA (anthrax) and F1 and V antigens (plague) are potential correlates with protection. This parameter was used to select potential attenuated ΔphoP/Q *Salmonella typhimurium* constructs expressing PA, F1, V, F1-V (fusion protein), or fragments of PA and V from Asd^+ balanced-lethal plasmids to maintain stable antigen-producing Salmonella vectors in the absence of antibiotic

selection. Various plasmid expression vectors were evaluated that either secreted the antigen, placed the antigen in the outer membrane, or expressed the antigen in the bacterial cell cytoplasm.

[0013] *Salmonella typhimurium* M020 is being developed by AVANT as an oral, single-dose, Plague vaccine candidate. Strain M020 was attenuated through the partial deletion of the *Salmonella phoP/Q* virulence regulon. A deletion in the *asd* gene was subsequently introduced to support a balanced-lethal plasmid maintenance system. *Salmonella typhimurium* M020 harbors a multi-functional plasmid that encodes Asd and a genetic fusion of the *Y. pestis* F1 and V antigens ("F1-V"). Strain M020 was genetically stable during laboratory growth and expressed moderate levels of F1-V that remained localized in the bacterial cytoplasm.

[0014] To evaluate immunogenicity, mice were orally inoculated with frozen inocula of 1×10^9 CFU on days 0 and 14. Retained inocula samples were evaluated by Western blot after feeding to demonstrate the desired antigen was still being expressed. Naïve mice and mice inoculated with a live bacterial vector expressing no antigen were included as controls. Serum was collected at day zero prior to immunization from 10 mice and again at 2 and 4 weeks post-boost and evaluated for IgG antibodies against Salmonella vector and target antigen.

[0015] Two strains were found to induce high levels of serum IgG specific to the expressed heterologous antigen. The strains were M020, which expresses soluble F1-V in the bacterial cell cytoplasm and M023, which expresses soluble V at extremely high levels in the bacterial cell cytoplasm. End-point titers (reciprocal of highest dilution above 0.1 OD450 as measured by ELISA) for serum IgG specific to V antigen for M020 vaccinated mice ranged from 100-1600 at 2 weeks post-boost and 400-6400 at 4 weeks post-boost. End-point titers for M023 ranged from 1600-6400 at 2 weeks post-boost and 800-25600 at 4 weeks post-boost. End-point titers for serum IgG specific to F1-V for M020 ranged from 100-6400 at 2 weeks post-boost and 400-6400 at 4 weeks post-boost.

[0016] Improved results were observed in a recently developed rabbit immunogenicity model. New Zealand White rabbits given a single intra-gastric dose of *S. typhimurium* M020 (approximately 2×10^{10} cfu) showed significant antibody titers against F1 (800-3200), V antigen (6400-51,200), and F1-V (12,800-51,200) on day 20 following immunization (results of an intra-gastric M020 boost are pending). Collectively, these results demonstrate that F1-V expressed by an attenuated strain of *S. typhimurium* was recognized by immunized mice and rabbits eliciting the production of high levels of antibodies.

[0017] Currently six additional candidates are undergoing testing. Based on these early comparisons the most promising results were obtained from cytoplasmic localized F1-V and V antigens.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Figure 1 is a chart listing the *S. typhimurium* strains constructed and tested as anthrax and plague vaccine candidates, showing the cell bank ID number, the strain and plasmid ID number, the particular antigen expressed, and the area in which it is localized (e.g., the cytoplasm, secreted, etc.), the plasmid replicon/ori, and the genotype of the particular strain.

[0019] Figures 2a-c are photographs showing the antigen expression (on 12-15% SDS-PAGE gels) in primary inoculum of each evaluated strain. Depending upon the candidate, overnight cultures, mid-log cultures, TCA precipitants, periplasmic fractions, outer membrane fractions, or soluble and insoluble fraction of whole cell lysates were prepared. The following designations represent the particular sample preparation: "WCL" represents whole cell lysate preparations; "TCA" represents trichloroacetic acid precipitated protein fractions; "OMP" represents outer membrane protein fractions; "S" represents soluble protein fractions; and "I" represents insoluble protein fractions.

[0020] Figures 3a-c are charts showing the mouse serum IgG response specific to *S. typhimurium* LPS antigen, indicating that the immune systems of the test mice had been exposed to the respective *S. typhimurium* vaccine candidates. The charts illustrate the particular *S. typhimurium* anthrax and/or plague vaccine candidates, the strain ID number, the particular attenuating mutation in the *S. typhimurium*, the heterologous antigen expressing plasmid(s), and the Optical density (OD) measurement at 450nm (the wavelength at which anti-*S. typhimurium* LPS antibodies are absorbed).

[0021] Figure 4 is a plasmid map of plasmid pMEG-1621, which expresses F1-V fusion protein antigen localized in the cytoplasm when used to transform *S. typhimurium*.

[0022] Figure 5 is a plasmid map of plasmid pMEG-1707, which expresses F1 antigen protein localized in the cytoplasm when used to transform *S. typhimurium*.

[0023] Figure 6 is a plasmid map of plasmid pMEG-1692, which expresses the V antigen protein localized in the cytoplasm when used to transform *S. typhimurium*.

[0024] Figure 7a is a plasmid map of plasmid pMEG-1967, which expresses the F1 and V antigen proteins localized in the cytoplasm when used to transform *S. typhimurium*.

[0025] Figure 7b is a plasmid map of plasmid pMEG-1968, which also expresses the F1 and V antigen proteins localized in the cytoplasm when used to transform *S. typhimurium*, but with the pUC replicon.

[0026] Figure 8 is a chart showing the OD values and endpoint titers to an F1-V fusion antigen induced by five attenuated *S. typhimurium*-vectored *Y. pestis* candidates.

[0027] Figure 9 is a chart showing the OD values and endpoint titers to F1 induced by M022, M020, M048, and M049.

[0028] Figure 10 is a chart showing the OD values and endpoint titers to V antigen induced by M020, M023, M048, and M049 attenuated *S. typhimurium*-vectored *Y. pestis* candidates.

[0029] Figure 11 is a chart showing the optimized plague study designed to evaluate the combination of vaccine candidates and the timing of vaccination.

[0030] Figure 12 is a chart showing the serum IgG F1 and V endpoint titer data at 2 and 5 weeks post-boost.

[0031] Figure 13 is a chart showing the serum IgG F1-V and LPS endpoint titer data and OD values at 2 and 4 weeks post-boost.

[0032] Figure 14 is a chart showing the serum IgG F1, V, and F1-V rabbit titer data at day 20, 29, 43 and 13 days post-boost.

DETAILED DESCRIPTION

[0033] The potential exposure of humans to bioterrorism agents, such as *Bacillus anthracis* and *Yersinia pestis*, has prompted the investigation of new vaccines to counteract these threats. Employing attenuated bacterial vectors to deliver protective antigens, such as the protective antigen (PA) of *B. anthracis* and/or the F1, V, or F1-V (fusion protein) antigens of *Y. pestis*, are presented herein. Delivery of heterologous antigens by attenuated bacteria, especially *S. typhimurium*, is disclosed, and the influence of antigen localization within the delivering cell is shown to effect vaccine efficacy.

[0034] Attenuated ΔphoP/Q *Salmonella typhimurium* constructs expressing PA, F1, V, F1-V (fusion protein) or fragments of PA and V from Asd^+ balanced-lethal plasmids (to maintain stable antigen producing Salmonella vectors in the absence of antibiotic selection) are presented herein. Various plasmid expression vectors were evaluated that either secreted the antigen, placed the antigen in the outer membrane, or expressed the antigen in the bacterial cell cytoplasm.

Strain Construction:

Basic Strategy:

[0035] Primers for PCR were designed for each target antigen or fragment with appropriate restriction enzyme sites designed within the flanking 5' and 3'ends.

[0036] PCR products (i.e., inserts) and plasmid vectors were digested with the appropriate restriction enzymes.

[0037] PCR products and plasmid vectors were cleaned and ligated overnight at 17°C.

[0038] Ligated products were drop dialyzed and electroporated into MGN055, an *E.coli* host strain, for screening.

[0039] Plasmids were screened by either PCR or restriction enzyme digestion for insert of interest.

[0040] Plasmids confirmed to contain the proper insert were electroporated into MGN5670.

Evaluation of Expression:

Basic Strategy:

[0041] Depending upon the candidate, overnight cultures, mid-log cultures, TCA precipitants, periplasmic fractions, outer membrane fractions,or soluble and insoluble fraction of whole cell lysates were prepared.

[0042] A portion of each preparation was run on 12-15% SDS-PAGE gels.

[0043] One gel was stained with Gel Code, a coomassie stain.

[0044] One gel was blotted to nitrocellulose and a western blot analysis was performed with the appropriate antiserum to verify expression.

Preparation of Inoculum from Frozen Cell Banks:

[0045] A standing overnight culture was started by thawing 1 vial of the frozen cell bank at 37° C. 5µl of the thawed cells was used to inoculate 10ml of LB (Luria-Bertani). Cultures were then incubated as standing cultures at 37° C overnight. In addition, 95mL of LB was placed in a 500-ml flask and pre-warmed at 37° C overnight.

[0046] The following day, cultures were diluted 1:20 by adding 5ml to 95-ml of pre-warmed LB. Cultures were incubated at 37° C in a shaking incubator and monitored until OD600 values reached 1.0.

[0047] The culture was centrifuged, and the pellet resuspended in 5ml peptone glycerol. This gave a titer of approximately 2×10^{10} CFU/ml, or 1×10^{9} CFU in a 50µl dose. The inoculum was then distributed into 1ml aliquots in pre-labeled cryovials, titered, and stored at -70° C. After at least 24 hours in the freezer, one vial was removed and thawed at 37° C to check the titer after a freeze/thaw cycle.

[0048] On the day mice were inoculated, one vial of inoculum was thawed at 37° C, 100µl was removed for titering, and 50µl volume fed to 5-10 mice. Remaining inoculum was used to start overnight cultures, which were used to evaluate the expression of target antigen.

Evaluation of Candidates in BALB/c mice:

[0049] Female MSP BALB/c mice 6-8 weeks of age from Taconic were acclimated for 1 week.

[0050] 10 naïve mice were heart bled for background serum titers. In some studies, all mice were pre-bled by tail vein puncture.

[0051] On days 0 and 14 of the experiment, the inoculum was thawed, titered, and a 50 ml volume was given to each mouse by pipette feeding.

[0052] Mice were monitored daily.

[0053] At 2 and 4 weeks post-boost, heart blood was drawn from a set five mice from each group.

[0054] Blood was allowed to clot and spun.

[0055] Serum was collected and stored at -20° C until evaluated in ELISA assays.

ELISA Assays:

LPS:

[0056] *S. typhimurium* LPS was prepared in 0.2% TCA and used to coat Immulon I-flat bottom 96-well plates. Incubate plates at 37° C for 2 hours.

[0057] Serum was diluted 1:100 for initial readings or 1 to 2 dilutions for endpoint titers.

[0058] Plates were washed with TBS/0.1% Tween 20 and diluted serum added to samples in duplicate, then incubated for 1 hour at 37° C.

[0059] Plates were washed with TBS/0.1% Tween 20. Appropriate anti-mouse diluted detection antibody conjugated with Peroxidase or Alkaline Phosphatase was added and incubated at 37° C for 1 hour. Plates were washed with TBS/0.1%Tween 20.

[0060] Plates were developed with either BluePhos or TMP kits. The reaction was allowed to develop to known standard, and then stopped with either 2.5%EDTA tetrasodium salt or 1M phosphoric acid (H_4PO_4).

Protein:

[0061] Plates coated with 1-10 μg of protein per 1 ml in PBS were stored overnight at 4° C.

[0062] Plates were dumped the following day and blocking solution (2% casein filler) was added incubated for 30 minutes at room temperature.

[0063] Serum samples were diluted in 2% casein and added to wells in duplicate, incubated for 2 hours at room temperature.

[0064] The plates were washed and the detection method outlined above followed.

Results:

[0065] Serum IgG endpoint titers (reciprocal of highest dilution above 0.1 OD450 as measured by ELISA) specific to the heterologous antigen(s) have been used to evaluate 22 potential attenuated *S. typhimurium*-vectored anthrax and plague candidates. To date, this project has identified five plague candidates. M020 vaccinated mice developed endpoint titers at 2- and 4- weeks post-boost specific to: (i)V antigen ranging from 100-1600 and 200- 3200, (ii) F1 antigen ranging from 0-400, and (iii) F1-V fusion ranging from 100-6400 and 400-6400. M023 vaccinated mice developed endpoint titers at 2- and/or 4-weeks post-boost specific to: (i) V antigen ranging from 800-3200 and 1600-51200 and (ii) F1-V fusion ranging from 3200-12800 and 3200-102400. M022 vaccinated mice developed endpoint titers at 2- and 4-weeks post-boost specific to: (i) F1 antigen ranging from 0-1600 and 400-6400 and (ii) F1-V fusion ranging from 0-1600 and 400-6400. At 4-weeks post-boost, M048 vaccinated mice developed endpoint titers specific to: (i) V antigen ranging from 400-25600, (ii) F1 antigen showed no response, and (iii) F1-V fusion ranging from 1600-102400. At 4-weeks post-boost, M049 vaccinated mice developed endpoint titers specific to: (i) V antigen ranging from 0-1600, (ii) F1 antigen ranging from 200-6400, and (iii) F1-V fusion ranging from 400-6400. Based on these early comparisons, promising results were obtained from cytoplasmic localized F1-V fusion-expressing constructs, V-expressing constructs, and F1- and V-expressing constructs.

[0066] New Zealand White rabbits given a single intra-gastric dose of *S. typhimurium* M020 (approximately 2×10^{10} cfu) showed significant antibody titers against F1 (800-3200), V antigen (6400-51,200), and F1-V (12,800-51,200) on day 20 following immunization (results of an intra-gastric M020 boost are pending). Collectively, these results demonstrate that F1-V expressed by an attenuated strain of *S. typhimurium* was recognized by immunized mice and rabbits eliciting the production of high levels of antibodies.

ABSTRACT OF THE DISCLOSURE

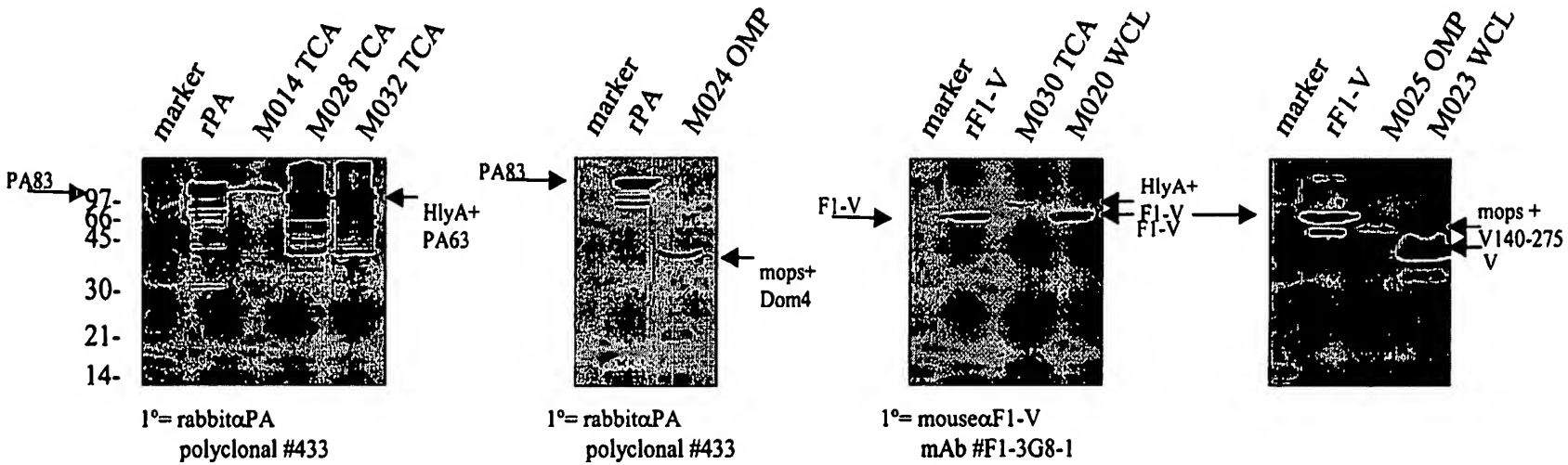
Oral vaccines against anthrax (*Bacillus anthracis*) and plague (*Yersinia pestis*) based on the delivery of protective antigens by attenuated, live bacterial vectors derived from *Salmonella enterica* (serovar Typhimurium) are disclosed.

Figure 1: *S. typhimurium*-Vectored Anthrax and Plague Vaccine Candidates

Cell Bank	Strain	Antigen/ Location	Replicon	Genotype
M028	MGN7063 (pMEG-1773)	HlyA::PA63 Secreted	pUC Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M032	MGN7078 (pMEG-1773)	HlyA::PA63 Secreted	pUC Asd+	*Δcya-27 Δcrp-28 ΔasdA16*
M024	MGN6830 (pMEG-1668)	OmpS::PA(Dom4) Surface	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M030	MGN7067 (pMEG-1777)	HlyA::F1-V Secreted	pUC Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M020	MGN6795 (pMEG-1621)	F1-V Cytoplasm	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M025	MGN6993 (pMEG-1740)	OmpS::V(140-275) Surface	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M023	MGN6973 (pMEG-1692)	V Cytoplasm	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M035	MGN7123 (pMEG-1820)	Inp::HAR+Dom4 of PA Surface	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M039	MGN7237 (pMEG-1852)	PpagC+HlyR+HlyC+HlyA::PA63(long) Secreted	pBR Asd+	*Δcya-27 Δcrp-28 ΔasdA16*
M040	MGN7265 (pMEG-1862)	PpagC+HlyA::PA63(short) Secreted	pBR Asd+	*Δcya-27 Δcrp-28 ΔasdA16*
M041	MGN7269 (pMEG-1867)	Dom3,4 of PA Cytoplasm	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M036	MGN7152 (pMEG-1811)	V::Dom3,4 of PA Cytoplasm	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M037	MGN7176 (pMEG-1823)	HlyR+HlyC+HlyA+HlyB+HlyD::V Secreted	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M022	MGN6928 (pMEG-1707)	F1 Cytoplasm	pUC Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M044	MGN7428 (pMEG-1923)	PpagC+PA63 Cytoplasm	pBR Asd+	*Δcya-27 Δcrp-28 ΔasdA16*
M045	MGN7416 (pMEG-1934)	F1 Operon Surface	pSC101 Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M047	MGN7283 (pMEG-1612) (+pMEG-1841 chaperone)	Inp::PA63 Surface	pBR Asd+ p15A GlnA+	*ΔphoPQ956 ΔasdA19(pBAD.C2) ΔglnA1*
M048	MGN7483 (pMEG-1967)	F1 and V Cytoplasm	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M049	MGN7484 (pMEG-1968)	F1 and V Cytoplasm	pUC Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M052	MGN7503 (pMEG-1985)	HAR+Dom4 Cytoplasm	pBR Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M054	MGN7526 (pMEG-1992)	PelB::PA83 (optimized sequence) Periplasm	Runaway Asd+	*ΔphoPQ956 ΔasdA19(pBAD.C2)*
M055	MGN7532 (pMEG-1994)	PelB::PA83 (optimized sequence) Periplasm	pBR GlnA+	*ΔphoPQ956 ΔglnA1*
M019	MGN6476 (pYA3342)	Vector only negative control	pBR	*ΔphoPQ956 ΔasdA19(pBAD.C2)*

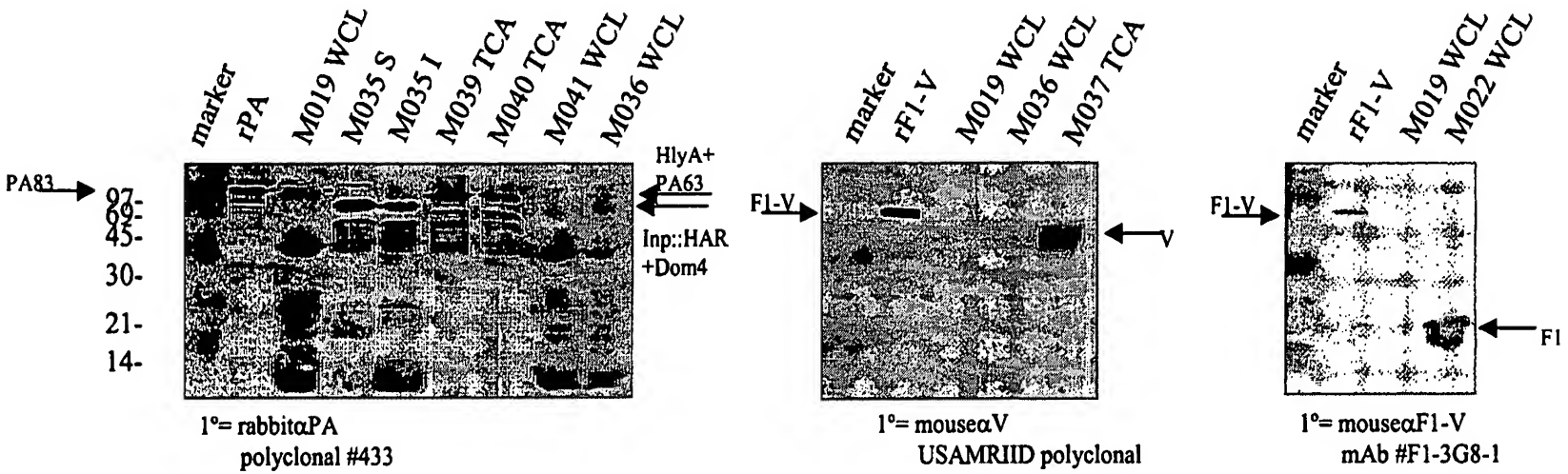
Highlighted candidates induced immune responses in mice. See data below.

Figure 2a.: Antigen Expression in Primary Inoculum of each evaluated strain



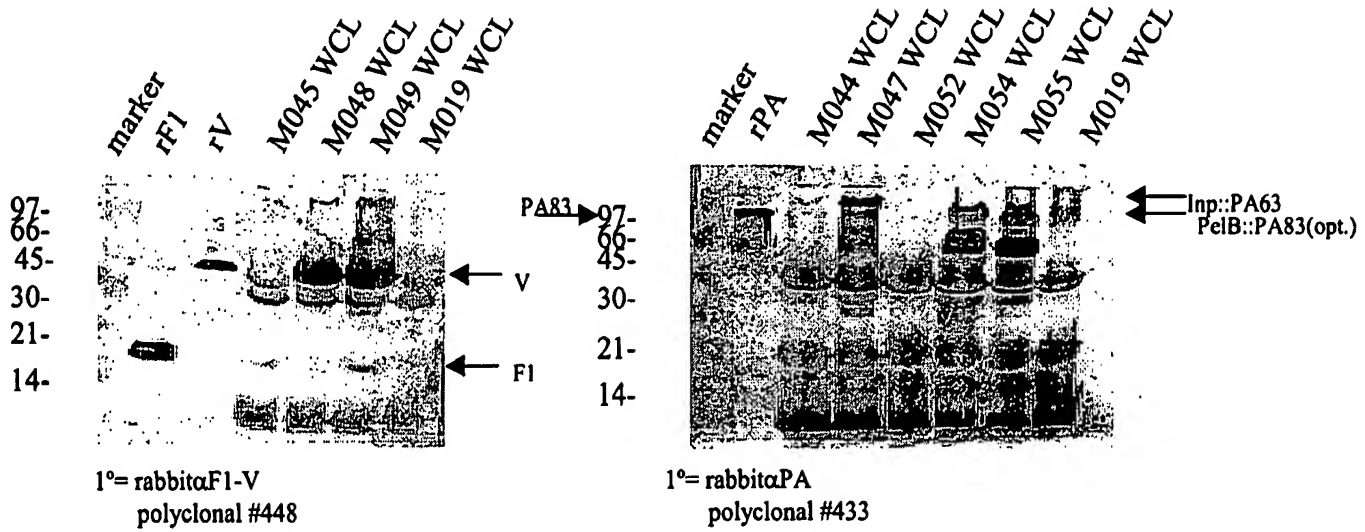
WCL= Whole Cell Lysate
TCA= TCA Precipitated Protein
OMP= Outer Membrane Protein
S= Soluble Protein
I= Insoluble Protein

Figure 2b.: Antigen Expression in Primary Inoculum of each evaluated strain



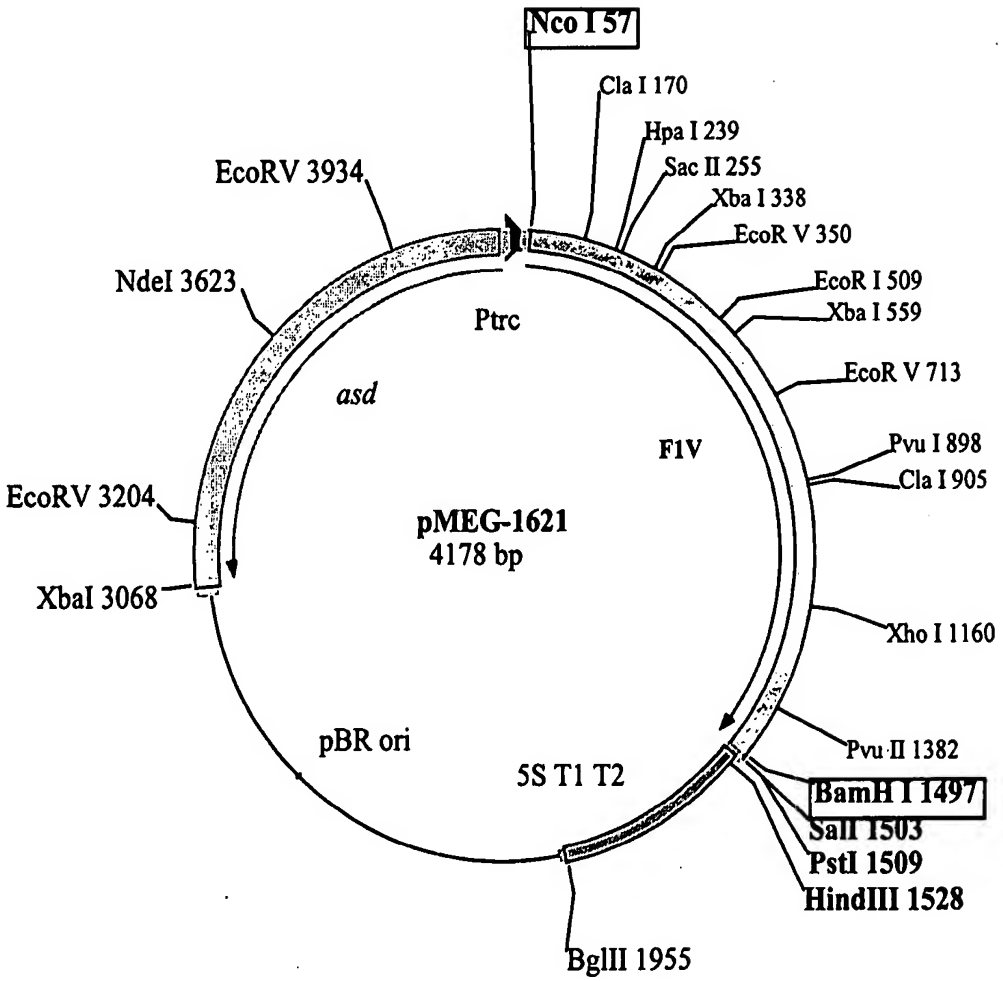
WCL= Whole Cell Lysate
TCA= TCA Precipitated Protein
OMP= Outer Membrane Protein
S= Soluble Protein
I= Insoluble Protein

Figure 2c.: Antigen Expression in Primary Inoculum of each evaluated strain



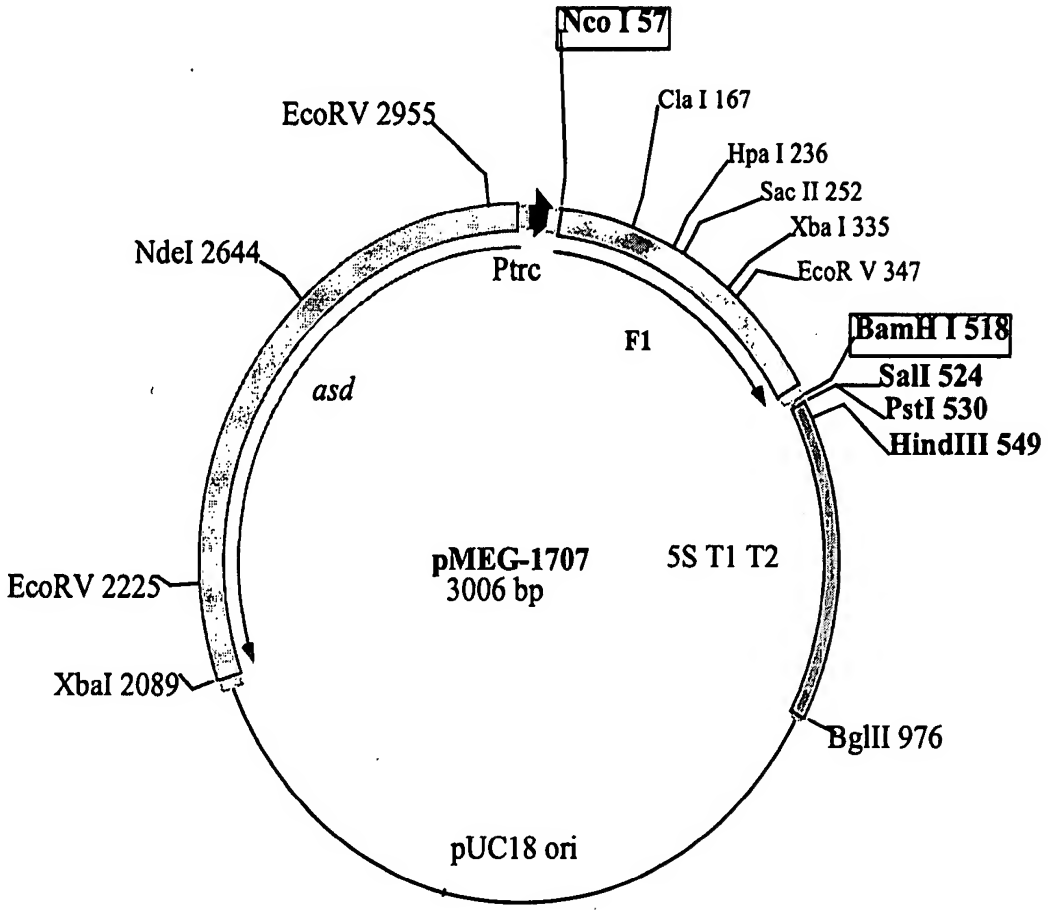
WCL= Whole Cell Lysate
TCA= TCA Precipitated Protein
OMP= Outer Membrane Protein
S= Soluble Protein
I= Insoluble Protein

Figure 4: Plasmid pMEG-1621



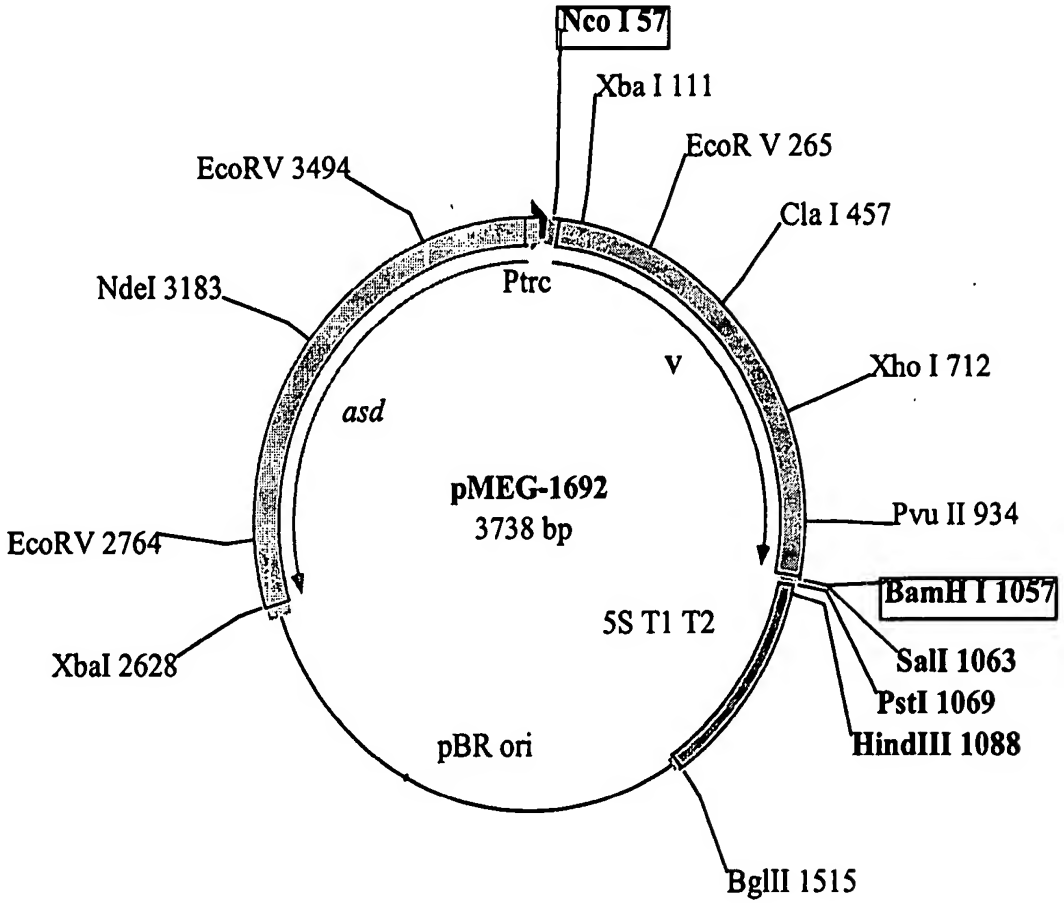
M020

Figure 5: Plasmid pMEG-1707



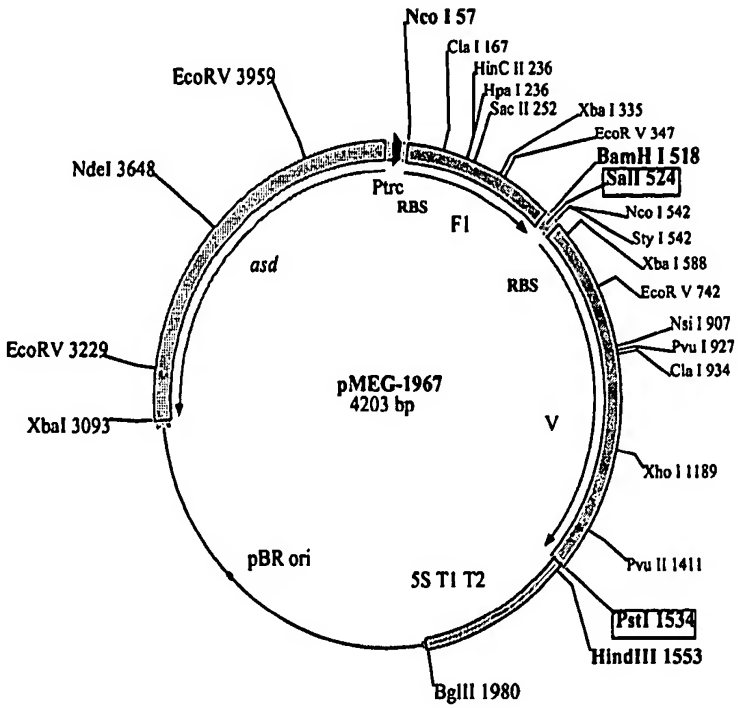
M022

Figure 6: Plasmid pMEG-1692



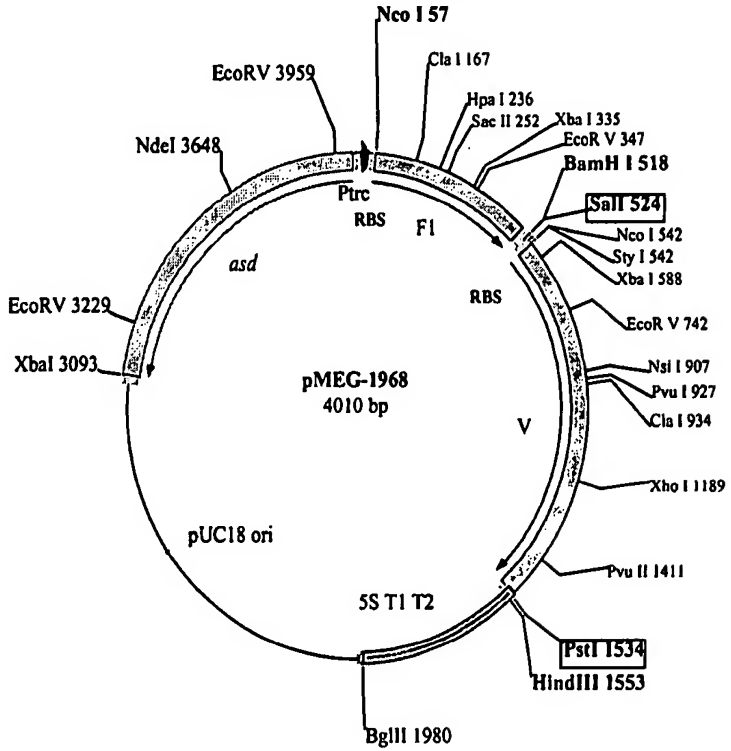
M023

Figure 7a. Plasmid pMEG-1967



M048

Figure 7b. Plasmid pMEG-1968



M049

Figure 8: OD Values and Endpoint titers to an F1-V fusion antigen induced by five attenuated *S. typhimurium*-vectored *Y. pestis* candidates

	2 weeks post boost			4 weeks post boost		
	Mouse ID	OD 630 value	Titer	Mouse ID	OD 630 value	Titer
MO20 F1-V	296	1.812	3200	301	1.804	6400
	297	1.473	1600	302	0.818	800
	298	1.790	6400	303	1.531	3200
	299	0.071	100	304	0.679	400
	300	1.937	6400	305	2.137	6400
Avg.		1.417	3540		1.393	3440
	2 weeks post boost			4 weeks post boost		
M023 V	316	1.030	3200	321	1.407	12800
	317	1.458	6400	322	0.879	3200
	318	1.270	6400	323	0.987	3200
	319	1.522	12800	324	1.787	102400
	320	1.339	12800	325	1.989	51200
Avg.		1.323	8320		1.409	34560
	2 weeks post boost			4 weeks post boost		
M022 F1	521	0.077	200	526	0.492	1600
	522	0.288	800	527	0.207	400
	523	-0.027	<100	528	1.927	800
	524	0.736	1600	529	1.451	3200
	525	0.524	800	530	2.080	6400
Avg.		0.319	700		1.231	2480
	2 weeks post boost			4 weeks post boost		
MO48 F1+V pBR	No data of this timepoint			751	1.353	12800
				752	1.629	51200
				753	1.644	102400
				754	1.569	25600
				755	0.559	1600
Avg.					1.350	38720
	2 weeks post boost			4 weeks post boost		
M049 F1+V pUC	No data for this timepoint			756	1.223	6400
				757	1.333	6400
				758	1.042	6400
				759	0.096	400
				760	0.286	400
Avg.					0.796	4000

Figure 9: OD values and endpoint titers to F1 induced by M022, M020, M048, and M049

	2 weeks			4 weeks		
Group ID	Mouse #	OD value	Endpt Titer	Mouse #	OD values	Endpt. Titer
M022 F1 Cytoplasmic ?phoP	521	0.346	200	526	2.254	800
	522	1.304	800	527	0.838	400
	523	0.033	100	528	2.287	800
	524	2.082	1600	529	3.868	3200
	525	1.755	800	530	3.868	6400
	Avg.	1.104	700	Avg.	2.623	2320
M020 F1-V Cytoplasmic ?phoP	296	0.905	200	301	0.061	<100
	297	0.529	200	302	0.11	100
	298	0.818	200	303	0.121	100
	299	0.302	100	304	0.09	<100
	300	1.359	400	305	0.826	200
	Avg.	0.511	220	Avg.	242	80
M048 F1+V pBR	No 2 week data taken.			751	0.023	<100
				752	0.028	<100
				753	0.030	<100
				754	0.036	<100
				755	0.021	<100
				Avg.	0.026	<100
M049 F1+V pUC	No 2 week data taken.			756	1.779	6400
				757	1.304	6400
				758	0.688	1600
				759	0.125	200
				760	0.322	400
				Avg	844	3000

Figure 10: OD Values and endpoint titers to V antigen induced by M020, M023, M048, and M049:attenuated *S. typhimurium*-vectored *Y. pestis* candidates

	2 weeks post boost			4 weeks post boost		
	Mouse ID	OD 630 value	Titer	Mouse ID	OD 630 value	Titer
MO20 F1-V	296	1.581	1600	301	1.929	800
	297	1.555	800	302	0.706	200
	298	1.628	1600	303	1.884	1600
	299	0.053	100	304	0.793	200
	300	1.766	1600	305	2.578	3200
Avg.		1.316	1140		1.578	1200
	2 weeks post boost			4 weeks post boost		
M023 V	316	1.450	800	321	2.160	3200
	317	1.914	3200	322	1.601	1600
	318	1.784	1600	323	1.940	3200
	319	1.848	3200	324	2.936	51200
	320	1.834	1600	325	2.698	12800
Avg.		1.766	2080		2.267	14400
	2 weeks post boost			4 weeks post boost		
M048 F1+V pBR	No data for this timepoint			751	1.821	3200
				752	2.318	6400
				753	2.436	25600
				754	1.826	12800
				755	0.775	400
Avg.					1.835	9680
	2 weeks post boost			4 weeks post boost		
M049 F1+V pUC	No data for this timepoint			756	0.334	100
				757	0.429	200
				758	0.973	1600
				759	-0.023	<100
				760	1.161	100
Avg.					0.574	400

Figure 11: Optimized Plague Study- Evaluating Combination of Candidates and Timing of Vaccination

Strain or combination of strains:	1° inoculation:	Boost:	Blood draw times:	Testing for serum responses to :	Number of animals:
F1-V	Day 0	Day 14	Weeks 4 and 6	Salmonella LPS and F1 and V	10
F1-V	Day 0	Day 28	Weeks 4 and 6	Salmonella LPS and F1 and V	10
F1 and V strains together	Day 0 both	Day 14 both	Weeks 4 and 6	Salmonella LPS and F1 and V	10
F1 and V strains together	Day 0 for F1 Day 3 for V	Day 14 F1 Day 17 V	Weeks 4 and 6	Salmonella LPS and F1 and V	10
F1-V and F1	Day 0 F1-V	Day 14 F1	Weeks 4 and 6	Salmonella LPS and F1 and V	10
F1-V and V	Day 0 F1-V	Day 14 V	Weeks 4 and 6	Salmonella LPS and F1 and V	10
F1-V and F1 and V	Day 0 F1-V	Day 14 F1 Day 15 V	Weeks 4 and 6	Salmonella LPS and F1 and V	10
F-V	Day 0, 3	Day 14	Weeks 4 and 6	Salmonella LPS and F1 and V	10
Vector only	Day 0	Day 14	Weeks 4 and 6	Salmonella LPS and F1 and V	10
Naïve	N/A	N/A	Background: prior to inoculation	Salmonella LPS and F1 and V	10

Strains tested:

F1-V= M020= MGN6795, *?phoP/Q S. typhimurium* with pMEG1621; pBR *asd*$^+$ vector expressing F1-V

F1= M022= MGN6928, *?phoP/Q S. typhimurium* with pMEG1707; pUC *asd*$^+$ vector expressing F1

V= M023= MGN6973, *?phoP/Q S. typhimurium* with pMEG1692; pBR *asd*$^+$ vector expressing F1-V

Vector only= M019= MGN6476, *?phoP/Q S. typhimurium* with pYA3342; pBR a*sd*$^+$ vector

Figure 12: Serum IgG F1 and V Endpoint Titer Data at 2 and 4 weeks Post-boost

ELISA	F1-V Day 0, Day14	F1-V Day 0, Day 28	F1 and V Day 0, Day14	F1 Day 0, 14 V Day 3, 17	F1-V Day 0 F1 Day 14	F1-V Day 0 V Day 14	F1-V Day 0 F1 Day 14 V Day 15	F1-V Day 0, Day 3, Day 14	Vector Only	Naive
F1 Endpoint 2 weeks Post-boost	200	<12.5	3200	1600	800	50	12800	50	<12.5 (pooled serum)	<12.5 (pooled serum)
	400	<12.5		6400	200	<25	800	400		
	100	25	6400	1600	50	50	200	400		
	200	<12.5	12800	800	<25	200	400	200		
	400	12.5	1600	1600	400	400	800	100		
F1 Endpoint 4 weeks Post-boost	100	800	12800	800	800	25	50	800	<25 (pooled serum)	<25 (pooled serum)
	100	400	6400	50	100	<25	50	800		
	25	200	12800	3200	100	25	25	800		
	50	100	6400	51200	800	<25	100	100		
	<25	800	25600	25600	800	<25	400	200		
V Endpoint 2 weeks Post-boost	800	<12.5	6400	6400	6400	6400	51200	800	<12.5 (pooled serum)	<12.5 (pooled serum)
	800	100		1600	800	25600	25600	1600		
	800	800	6400	6400	800	12800	6400	1600		
	3200	<12.5	3200	1600	100	6400	25600	800		
	25600	400	12800	1600	200	3200	6400	800		
V Endpoint 4 weeks Post-boost	12800	12800	800	3200	800	1600	3200	6400	<25 (pooled serum)	<25 (pooled serum)
	25	12800	1600	800	100	1600	3200	3200		
	100	1600	25	400	400	1600	400	200		
	400	6400	12800	800	100	200	3200	400		
	400	12800	1600	6400	<25	1600	12800	1600		

*10 mice were inoculated as indicated then 5 were sacrificed at 2 weeks post-boost. Another 5 mice were then sacrificed at 4 weeks post-boost.

Figure 13: Serum IgG F1-V and LPS Endpoint Titer Data and OD values at 2 and 4 weeks Post-boost

F1-V Endpoint 2 weeks Post-boost	800	<12.5	12800	6400	3200	6400	25600	1600		
	800	100		6400	800	25600	25600	1600	<12.5 (pooled serum)	<12.5 (pooled serum)
	400	400	12800	6400	800	3200	6400	1600		
	1600	<12.5	12800	800	50	6400	25600	400		
	25600	200	12800	1600	200	6400	12800	800		
F1-V Endpoint 4 weeks Post-boost	25600	12800	12800	12800	3200	6400	25600	12800		
	200	12800	6400	800	200	6400	6400	6400	<25 (pooled serum)	<25 (pooled serum)
	400	1600	6400	6400	800	6400	800	800		
	400	6400	25600	51200	800	800	25600	800		
	400	12800	25600	25600	400	6400	204800	1600		
LPS 2 weeks Post-boost	0.763	0.011	0.856	0.299	0.784	0.912	0.917	0.226	0.179	0.001
	0.075	0.022	NA	0.999	0.459	0.955	1.349	0.673	0.790	0.049
	0.741	0.692	0.577	0.293	0.417	0.186	0.683	0.355	0.316	0.084
	0.567	0.030	0.560	0.549	0.179	0.447	0.815	0.193	0.140	0.070
	0.993	0.060	0.714	0.700	0.337	0.649	0.481	0.224	1.003	0.077
LPS 4 weeks Post-boost	1.144	1.627	0.550	1.630	1.246	0.434	0.634	0.947	0.340	0.007
	0.366	1.458	0.800	1.151	0.973	1.182	0.547	0.968	0.897	0.003
	0.385	0.642	0.401	1.308	0.249	0.684	0.639	0.534	0.050	0.006
	0.365	1.782	0.241	0.536	0.227	1.829	0.095	0.481	0.114	0.031
	1.049	1.841	1.241	1.676	0.190	0.691	1.184	0.707	0.240	0.005

Figure 14: Immunogenicity of *S. typhimurium* vectoring F1-V antigen in a rabbit model

Serum IgG Titer[1] to:	Rabbit #	Prebleed	Day 20	Day 29	Day 43	13 days post-boost[3]
F1	641	<100	800	800	1600	1600
	642	<100	3200	1600	1600	3200
	643	<100	3200	1600	1600	3200
	644	<100	3200	6400	25600	25600
	GMT[2]	**<100**	**2263**	**1902**	**3200**	**4525**
V antigen	641	<100	6400	3200	6400	25600
	642	<100	6400	6400	12800	51200
	643	<100	25600	6400	6400	51200
	644	<100	51200	12800	25600	51200
	GMT	**<100**	**15222**	**6400**	**10763**	**43054**
F1-V	641	<100	12800	12800	6400	25600
	642	<100	51200	51200	25600	51200
	643	<100	12800	25600	12800	51200
	644	<100	25600	51200	51200	102400
	GMT	**<100**	**21527**	**30444**	**18102**	**51200**

[1]Reciporcal dilution; [2]geometric mean titer; [3]animals boosted orally with 2 x 10^{10} cfu M020 on day 44

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/041282

International filing date: 09 December 2004 (09.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/610,474
Filing date: 16 September 2004 (16.09.2004)

Date of receipt at the International Bureau: 09 February 2005 (09.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse